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A new automated human leukocyte antigen genotyping strategy to identify *DR-DQ* risk alleles for celiac disease and type 1 diabetes mellitus

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Abstract

Background: The risk for type 1 diabetes mellitus (T1DM) and celiac disease (CD) is related to human leukocyte antigen (HLA) *DQA1, DQB1* and *DRB1* loci. Unfortunately, HLA typing has been too difficult and costly for frequent use. Automated genotyping focused on risk alleles could provide access to HLA typing in diagnostic evaluations, epidemiological screening and contribute to preventive strategies.
Methods: A sequence specific primer amplification method requiring a total of four PCR reactions, one restriction enzyme digestion and a single electrophoretic step provides low to medium resolution typing of *DQA1, DQB1* and *DRB1* using Applied Biosystems 3730 DNA analyzer. The method was validated using 261 samples with genotypes determined using a reference method.
Results: Specific fluorescent *DQA1*, *DQB1* and *DRB1* amplicons were of expected size. Concordance with the reference method was 100% for *DQA1* and *DQB1* alleles and 99.8% for *DRB1* alleles.
Conclusions: We have developed a high throughput HLA typing method that accurately distinguishes risk alleles for T1DM and CD. This method allows screening of several thousand samples per week, consuming 32 ng of DNA template, low reagent volumes and minimal time for data review.
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Keywords: automation; celiac disease; HLA-*DR-DQ*-genotyping; PCR-SSP (sequence specific priming); type 1 diabetes mellitus.

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Introduction

The prevalence of type 1 diabetes mellitus (T1DM, OMIM %222100) is 0.05%–0.4% in the general population of Europe and the US (1–3), while the prevalence of celiac disease (CD, OMIM #212750) is close to 1% and possibly higher in Northern European countries (3–5). Both diseases occur during early childhood, cluster in families, and significantly affect health and family life-styles. Thus, risk assessment for siblings and other family members is frequently requested. Inter-action of environmental exposure with a genetic predisposition for autoimmune disease is well documented (6–10). The most important genes contributing to disease susceptibility are located in the human leukocyte antigen (HLA) class II region on chromosome 6p21.3. For both diseases, a very limited number of HLA *DQA1* and *DQB1* alleles appear to be prerequisite for an immune reaction.

Numerous studies have demonstrated that *DQB1*02* or **0302* expressed with *DQA1*05* or *DQA1*03*, respectively, confer a genetic risk for CD (11, 12). About 90%–95% of patients with CD carry the *DQB1*02* allele together with *DQA1*05* (encoded either in cis or trans), compared to a prevalence of 20%–30% in the general population (13). Most of the remaining 5%–10% have a DQ haplotype comprised of *DQA1*03* and *DQB1*0302* (11, 14, 15), and nearly no patients with CD lack both of these DQ risk combinations. Studies have shown an increased risk in individuals homozygous for *DQB1*02* (15–17) and an HLA-DQ risk gradient has been established for CD (Table 1) (18). Thus, screening for *DQB1*02* and **0302* allows very sensitive, but not specific, identification of subjects at risk (19).

The situation is somewhat more complex, but similar for T1DM, with risk also associated with *DQB1*0201* and **0302* alleles (20–22). However, this risk appears to be modified by the accompanying *DRB1* alleles (21, 23–26). A continuous gradient from highly predisposing to very protective haplotypes has been demonstrated in various studies (24, 27, 28). *DRB1*04-DQB1*0302* or *DRB1*03-DQB1*0201* are present in 90%–95% of patients who develop T1DM compared to 40%–45% of normal controls. Compound heterozygotes carrying both of these haplotypes confer an increased risk compared to homozygotes for either haplotype. *DRB1*15-DQB1*0602*, found in <1% of patients with T1DM and 20% of the general population, has a protective association with the disease (1, 29). Table 2 shows the HLA risk association with T1DM.

Table 1 CD risk associated with DQ combinations. Data from Megiorni et al. (18).

Haplotype 1		Haplotype 2		Risk
DQA1	DQB1	DQA1	DQB1	
0501	0201	0301	0302	1:7
0501	0201	x[a]	0201[b]	1:10
0301	0302	x[a]	0201[b]	1:24
x[a]	0201[b]	x[a]	0201[b]	1:26
0501	0201		0201[b] neg	1:35
0201	0202	0501	0301	1:35
0301	0302		0201[b] neg	1:89
x[a]	0201[b]		0201[b] neg	1:210
0501	0201 neg		0201[b] neg	1:1842
Other combinations				1:2518

[a]x differs from *DQA1 *0501*; [b]**02* is either *DQB1*0201* or **0202*.

Table 2 T1DM risk associated with common HLA-DR-DQ haplotypes (28).

	HLA			Mean P/C[a]
	DRB1	DQA1	DQB1	ratio
Highly predisposing	0405	0301	0302	7.15
	0401	0301	0302	6.23
	0402	0301	0302	5.10
	0301	0501	0201	3.72
	0404	0301	0302	2.93
	08	0401	0402	1.92
	0901	0301	0303	1.12
	01	01	0501	0.85
	04	0301	0301	0.73
	0701	0201	0202	0.66
	0403	0301	0302	0.64
	11	0501	0301	0.40
	0701	0201	0303	0.40
	13	01	0603	0.40
	1001	01	0501	0.32
Protective	14	01	0503	0.28
	15	01	0602	0.22

[a]P/C ratio is a ratio between the haplotype frequency inT1DM cases compared to the general population. The P/C ratio varies across population and ethnic regions, as they are a function of disease prevalence. For full risk assessment in different populations, see references (24, 27, 28).

In both CD and T1DM, the presence of a known risk allele indicates significantly increased relative risk, still lacking specificity. However, absence of all known risk alleles has a high negative predictive value – thus, obviating the need for additional diagnostic testing.

Testing for HLA risk can be performed using commercial kits such as Olerup SSP DQ (Genovision/Qiagen, Oslo, Norway), Delfia® HLA hybridization assay (Perkin Elmer Life and Analytic Sciences Wallac, Turku, Finland) or Dynal RELI™ SSO HLA typing kit (Dynal Biotech Ltd, Bromborough, UK). These methods require many reactions and/or multiple steps. We present a novel automated high-throughput PCR-SSP (sequence specific priming) method for HLA-*DRB1, DQA1* and *DQB1* genotyping, optimized for capillary electrophoresis using Applied Biosystems 3730 or 3130*xl* DNA Analyzers (Applied Biosystems, Foster City, CA, USA), with a specific focus on risk alleles for T1DM and CD.

Materials and methods

DNA samples

DNA samples derived from affected sib-pair families or trios were obtained following informed consent from participants from five continents enrolled in the type 1 Diabetes Genetic Consortium (T1DGC) (30). The project was approved by Ethical Review Boards in each participating country. A sample panel of 261 residual recoded DNA samples and 12 non-template controls (NTC) used for proficiency testing within the T1DGC HLA genotyping network were available with permission for use to validate this method. The samples have been genotyped by three accredited laboratories using a proprietary high-resolution PCR-based sequence specific oligonucleotide probe linear array (Roche Molecular Systems, Pleasanton, CA, USA). There was 100% interlaboratory consensus at these three loci, described by Mychaleckyj et al. (manuscript submitted).

HLA-*DQA1, DQB1* and *DRB1* typing

DNA sequences of the second exon of HLA class II genes were obtained from the IMGT/HLA database (31). Intronic sequences were obtained from genebank sequences listed in Bergström et al. (32) and Kotsch et al. (33). Sequence specific primers corresponding to exon 2 of the *DQA1* and *DQB1* genes, and to intron 1, exon 2 and intron 2 of the *DRB1* gene, were designed using Oligo 6.69 software (Molecular Biology Insights Inc, Cascade, CO, USA). Four different primer mixes were used, each containing at least one locus specific common primer labeled with a fluorescent dye specific for the locus (NED, a yellow fluorescent color, for *DQA1*, VIC, a green fluorescent color, for *DQB1* and FAM, a blue fluorescent color, for *DRB1*) and several allele specific primers. The common *DRB1* primer was placed 3′ to numerous microsatellite repeats to provide distinct class-specific amplicon sizes. Special care was taken to prevent co-amplification of the other existing *DRB* genes. All primers and primer mixes are listed in Table 3.

All primers were diluted to a concentration of 2.5 mM. Each PCR reaction contained 2 μL 4 ng/μL DNA, 0.6 μL 2.5 mM dNTP's (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), 0.6 μL GeneAmp® 10X PCR Buffer (500 mM KCl and 100 mM Tris-HCl, pH 8.3), 0.6 μL 25 mM $MgCl_2$, 0.05 μL AmpliTaq Gold™ DNA Polymerase (5 U/μL) (Applied Biosys-

Table 3 Primers and primer mixes used.

HLA primer mix	Primer name and specificity	Primer sequence (5′–3′)	T_m, °C[a]	Fragment length, bp[b]	Volume, μL
DQA1/DQB1 *05/*06	DQA1 all U *NED	[NED]GCCTCTT**CA**GGTGTAAACTTGTACCAGT[c]	73.2	–	0.40
	DQA1*01 L	GCCATGTTTCTCAGTGCACC	70.2	176	0.24
	DQA1*02 L	ATTGCGGGTCAAATCTAAGTCTGTG	72.9	154	0.10
	DQA1*03 L	CAAATCTCCTAAATCTGCGGAACAG	70.7	148	0.22
	DQA1*04/*05/*06 L	CTAAATTGTCTGAGAACAGGCAAACA	71.1	140	0.28
	DQA1*05 L	CGCAGACTGTTCAAGTTATGTTTTAGG	71.6	203	0.16
	DQB1*05/*06 L *VIC	[VIC]**CGACGACGCTCAC**CTCTCCT[d]	73.8	–	0.40
	DQB1*05 U	GTGCGGGGTGTGACCAGAC	73.7	227	0.15
	DQB1*06 U	GGAGCGCGTGCGTCTTGTAA	75.3	234	0.30
	DQB1*0601 U	GACGGAGCGCGTGCGTTA	75.4	236	0.15
DQB1 *02/*03/*04	DQB1*02/*03/*04 L *VIC	[VIC]GTCGTGCGGAGCTCCAACT	72.4	–	0.80
	DQB1*02 U	CGTGCGTCTTGTGAGCAGAA	72.2	201	0.32
	DQB1*04 U	GCGGGGTGTGACCAGATACA	72.8	198	0.16
	DQB1*0301/*0303 U	GCTGGGGCCGCCTGA	74.6	114	0.28
	DQB1*0301/*0304 U	GACAGCGACGTGGAGGTGTAC	71.5	149	0.10
	DQB1*0302/*0303 U	GAGCGC**A**TGCGTCTTGTGAC[e]	74.0	206	0.36
DRB1 mix I	DRB1 all L *FAM	[FAM]CTAAATGCTCACAGATGGCGCTCTCTCTC	78.9	–	0.80
	DRB1*01 U	CGTTTCTTGTGGCAGCTTAAGTT	70.3	[f]	0.40
	DRB1*04 U	CGGAGGCCGCTTCTGTA	69.6	[f]	0.40
DRB1 mix II	DRB1 all L *FAM	[FAM]CTAAATGCTCACAGATGGCGCTCTCTCTC	78.9	–	0.65
	DRB1*03/*11/*13/*14 U	CACGTTTCTTGGAGTACTCTACGTC	68.9	[f]	0.40
	DRB1*07 U	CACGTTTCCTGTGGCAGGG	73.5	[f]	0.40
	DRB1*08/*12 U	CACGTTTCTTGGAGTACTCTACGGG	72.6	[f]	0.30
	DRB1*09 U	CGGTATCTGCACAGAGGCAT	69.7	[f]	0.30
	DRB1*10 U	GGTTGCTGGAAAGACGCGTCC	76.8	[f]	0.30
	DRB1*15/*16 U	CGTTTCCTGTGGCAGCCTAAGAG	74.7	[f]	0.30

[a]The melting temperature (T_m) of the primer; [b]the calculated fragment length in basepairs (bp); [c]**CA**, mismatch against all DQA1 alleles; [d]**CGACGACGCTCA**, intron sequence; [e]**A**, mismatch; [f]see supplemental data Table 1.

tems), 2 μL primer mix (Table 3), and H_2O to a final volume of 6 μL.

PCR reactions were performed in barcoded 96- or 384-well plates (Abgene, Epsom, UK) using a GeneAmp PCR system 9700 (Applied Biosystems). The *DQB1 *02/*03/*04*, *DRB1* mix I and *DRB1* mix II were amplified following initial denaturation at 95°C for 10 min, with 45 cycles at 94°C for 1 min, 66°C for 30 s and 72°C for 1 min, and a final 10 min at 72°C. *DQA1/DQB1 *05/*06* was amplified using the same conditions, with the exception of a 64°C annealing temperature.

Following amplification, 7.5 μL of a digestion mixture containing 1.25 μL Buffer B+ (10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$, 0.1 mg/mL BSA), 0.1 μL of the restriction endonuclease Cfr42I (10 U/μL) (Fermentas Sweden, Helsingborg, Sweden) and 6.15 μL H_2O was added to each 6 μL of *DRB1* mix I PCR reaction and incubated at 37°C for 2 h. This enzyme cleaves *DRB1*04* subtypes associated with T1DM risk, i.e., **0401*, **0402*, **0404*, **0405* and **0408*, leaving the protective or neutral subtypes such as, **0403*, **0406* and **0407* undigested.

The *DQA1/DQB1 *05/*06* and *DQB1 *02/*03/*04* PCR reactions were diluted 1:10 in water. Eight μL of 2% GeneScan™ 500 LIZ™ standard in HiDi Formamide (Applied Biosystems) was dispensed into each well in a barcoded MicroAmp plate (Applied Biosystems). Next, 1 μL of each PCR reaction, i.e., diluted *DQA1/DQB1 *05/*06* and *DQB1 *02/*03/*04*, *DRB1* mix II and the digested *DRB1* mix I was added to each well. All mixing and dispensing steps were performed using a Multimek™ 96 robot (Beckman Coulter, Fullerton, CA, USA). The PCR products were denatured by a single heating step at 95°C for 5 min prior to electrophoresis using a 36-cm capillary with a 3730 DNA Analyzer and Fragment Analysis Run Module, POP-7 polymer and the G5 dye set. Analysis time for a 96 well plate is ~1 h. Some analyses were also performed using a 3130*xl* Genetic Analyzer, requiring 3 h per run.

Detected fragment sizes were correlated to the LIZ-labeled internal standard peaks on the x-axis. Each HLA locus is represented by a specific fluorescent dye and each analyzed allele is represented by a specific size. Automated allele calling was performed using GeneMapper v 3.0 (Applied Biosystems). All allele calls were verified by manual inspection of the GeneMapper images and haplotype checks were performed before final allele designation.

Results

The NED-labeled *DQA1* specific amplicons, VIC-labeled *DQB1* specific amplicons and FAM-labeled *DRB1* specific amplicons were easily distinguished by GeneMapper. Figure 1 shows the electropherograms produced by a sample with the genotype *DRB1*0401/*0301, DQA1*03/*05, DQB1*02/*0301*. True peaks could be distinguished readily from background noise by amplitude and position. Concordance with the reference method was 100% (522/522) for *DQA1* alleles, 100% (522/522) for *DQB1* alleles, and 99.8% (521/522) for *DRB1* alleles at the resolution level indicated in Supplemental data Table 2. No signal was seen for any NTC.

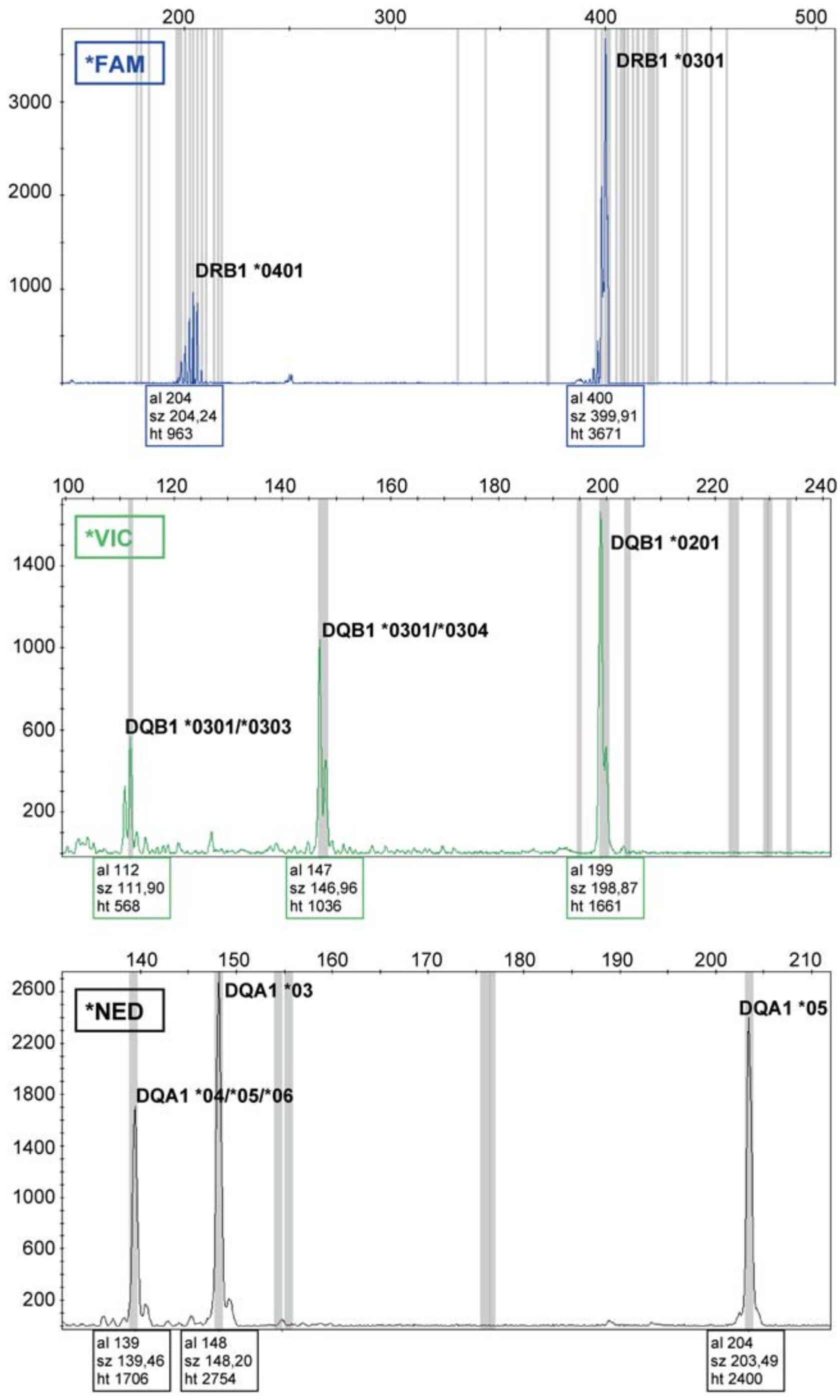

Figure 1 Electropherograms produced by a sample with the genotype *DRB1*0401/*0301*, *DQA1*03/*05*, *DQB1*02/*0301*. The position of each peak on the x-axis corresponds to the size of a PCR fragment. Peak heights correspond to the amount of detected PCR product. al, allele; sz, size; ht, height.

DQ allelic assignments

The sizes of all allele specific amplicons agreed with those expected and no overlap occurred, as shown in Supplemental data Figure 1. Although *DQA1*06* cannot be distinguished from *DQA1*04,* and heterozygous *DQA1*04/*05* and **05/*06* samples cannot be distinguished from homozygous *DQA1*05/*05*, such ambiguities can be resolved by haplotype analysis together with *DRB1* and *DQB1* data, choosing the most preferential allele identification as *DR* and *DQ* alleles exist in common although not entirely consistent linkage. Similarly, some heterozygous combinations of *DQB1*03* alleles appear ambiguous, but can be distinguished using haplotype association.

DRB1 allelic assignments

Whereas most *DRB1* alleles produced expected amplicon lengths, some displayed size variation due to a variable microsatellite in intron 2. Thus, a range of fragment sizes occurred with evident overlap between some allelic groups, see Supplemental data Figure 2. This method was able to distinguish the T1DM susceptible *DRB1*04* subtypes **0401, *0402*, **0404* and **0405* from the neutral or protective subtypes *DRB1 *0403* and **0407*. Of the 191 alleles typed as *DRB1*04* susceptible, one sample was *DRB1*0419*, which is very uncommon and the disease association is not known. *DRB1*0403*, **0407*, **07*, **09* and **10* alleles produced fragments of distinctive lengths. *DRB1*03*, **08*, and **16* alleles could be easily identified by referring to DR-DQ haplotypes. The *DRB1*11*, **12*, **13*, **14* and **15* alleles, which are generally neutral, were designated *DRB1**X as some overlap occurred. The T1DM protective *DRB1*1401* and **1501* alleles could be differentiated from the *DRB1**X group by size and haplotype association in 95% (82/86) of the cases. All analyzed haplotypes and the corresponding results, presumed by known linkage disequilibrium, are shown in Supplemental data Table 2.

Discussion

The major clinical importance of the HLA DR-DQ region is well established for CD and T1DM, and other associations within the HLA region are being investigated for rheumatoid arthritis, Morbus Addison and other diseases. Due to the extreme variability seen in this region, very large study populations are required to attain statistical power of association with other specific diseases.

To meet current local demands for an HLA method for the study of T1DM and CD, we developed a high throughput SSP genotyping method. Our method requires a maximum of 32 ng DNA templates, four PCR reactions, one restriction enzyme digestion and a single electrophoretic step using an automated DNA analyzer and automated allele assignment.

As specific risk alleles for these diseases are well established, high resolution of all neutral alleles is unnecessary. If a focus on other alleles is necessary, the method can be modified by exchange or addition of new primers and validation.

Since concordance of the *DQA1* and *DQB1* typing was 100%, and all *DQ* alleles associated with CD, i.e., *DQA1*05-DQB1*02* and *DQA1*03-DQB1*0302* could be distinguished from other alleles, this method is sufficiently sensitive and specific for clinical use to confirm or exclude the possibility of CD. Additionally, *DQB1 *02* homozygosity could be determined in the absence of another *DQB1* allele since at least one primer for every *DQB1* allele group (i.e., **02*, **03*, **04*, **05*, **06*) was included in the analysis.

The positive predictive value of the presence of these risk alleles is low. Thirty to forty percent of the Caucasian population carries at least one of these DQ combinations, and only 1%–4% of these individuals have CD (10). In contrast, the negative predictive value of their absence is $>$99.5% (12). Therefore, HLA genotyping can be useful for precluding the necessity for further invasive diagnostic testing or to exclude risk in relatives. It is estimated that most cases of CD remain undiagnosed, and untreated CD can cause long-term health problems. Thus, targeted screening in families of CD probands could identify such undiagnosed individuals and prevent lifelong symptoms and complications (4).

As for CD, the *DQB1*02* and/or **0302* alleles are present in nearly all T1DM cases. By including the restriction enzyme digestion step of *DRB1*04* and **01* PCR products, *DRB1*01* could be differentiated from other *DRB1* alleles and *DRB1* subtypes **0401*, **0402*, **0404*, **0405* associated with T1DM disease risk could be differentiated from **0403* and **0407* which are considered protective, even when present on the same ''high-risk'' *DQA1*03-DQB1*0302* haplotype (24). Also, the less common *DRB1*08-DQA1*04-DQB1*04* and *DRB1*01-DQA1*01-DQB1*05* risk alleles could be easily distinguished by this assay. Although allele frequencies and relative risks vary significantly in different populations and publications (24, 27, 28), essentially no cases lacking the above named alleles are seen in T1DGC cases (data unpublished).

A high-throughput screening assay such as that presented here could be useful in population studies such as ongoing prospective neonatal screening programs assessing T1DM risk (9, 34, 35). Also, analysis of HLA-*DR-DQ* alleles may be important for discriminating between subjects at high or intermediate risk from antibody positive individuals carrying protective haplotypes (36).

In our validation study using 261 samples from families with T1DM from five continents, *DRB1* typing made one incorrect identification only. Even though the sample panel originates from a T1DM study, 28 of the most common Caucasian haplotypes are represented in the dataset, covering all haplotypes with a frequency $>$0.3% in the general Caucasian population. In addition, several rare haplotypes are included in the dataset. However, we would caution that the efficiency of some markers in the screening protocol may depend on the population being studied, as allele frequencies and microsatellite repeats vary among different populations. It is therefore wise to validate this method against samples with known genotypes in different settings.

We developed an economical, high throughput HLA typing method that accurately distinguishes risk alleles for T1DM and CD with a degree of accuracy of 100% for *DQA1,* 100% for *DQB1* and 99.8% for *DRB1* genotyping. Simultaneous detection of *DRB1*, *DQA1* and *DQB1* alleles allows resolution of many potential ambiguities by reference to common haplotype associations. Although the method is not intended for matching of donors and recipients in the context of transplantation, it provides equal or better resolution compared with many available commercial kits and is well suited for clinical screening (e.g., as a negative predictor for CD or T1DM). The multiplex concept of this basic PCR-SSP capillary electrophoresis method is amenable to adaptation for many different disease associations.

By using locus specific fluorescent markers and capillary electrophoresis, multiple PCR products from all three loci can be automatically resolved by size, analyzed and typed in a single step. With a total requirement of 32 ng DNA template, HLA typing becomes possible using dried blood spots for DNA template. The low reagent costs and rapid interpretation allows analysis of high-risk alleles in epidemiological as well as clinical settings.

Supplementary data associated with this article can be found in the online version at: http://www.reference-global.com/doi/suppl/10.1515/CCLM.2009.346.

Conflict of interest

The authors claim no conflict of interest.

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